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Development of a Human Herpesvirus 6 Species-Specific Immunoblotting Assay

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In order to assess the full spectrum of human herpesvirus 6A (HHV-6A)- and HHV-6B-associated diseases, we sought to develop an HHV-6 species-specific serological assay based on immunoblot analysis. The immunodominant proteins encoded by open reading frame U11, p100 for HHV-6A (strain U1102) and 101K for HHV-6B (strain Z29), were selected to generate virus species-specific antigens. Recombinant p100 and 101K were produced in a prokaryotic expression system. The expression of these proteins was confirmed by using anti-His tag and 101K-specific monoclonal antibodies. HHV-6 species-specific antibodies were detected by immunoblotting in patient sera. Eighty-seven serum samples obtained from various subjects were utilized to determine the reliability of the method for clinical use. Ten of twelve exanthem subitum convalescent-phase sera reacted exclusively with 101K, whereas none of twelve acute-phase sera reacted with either protein. Two of three sera collected from HHV-6A-infected patients reacted with p100 and 101K. Although all five acute and convalescent-phase sera obtained from transplant recipients reacted exclusively with 101K, two of six convalescent-phase sera obtained from patients with drug-induced hypersensitivity syndrome reacted with both p100 and 101K. Of 38 sera obtained from healthy adults, 31 were positive for 101K antibody, while 4 reacted with both proteins. However, PCR analysis of peripheral blood mononuclear cells and saliva from these subjects did not detect HHV-6A DNA. In conclusion, this novel serological assay based on immunoblot analysis using recombinant HHV-6A p100 and HHV-6B 101K allowed us to discriminate between HHV-6A- and HHV-6B-specific antibodies.

Human herpesvirus 6 (HHV-6) is classified as two distinct virus species, designated HHV-6A and HHV-6B (2, 3, 20). Since the overall nucleotide sequence identity between the virus species is 90% (11, 17), distinguishing between the two species using serological analysis has proven difficult. It has been demonstrated that primary HHV-6B infection occurs in infancy and early childhood (31) and causes exanthem subitum (31, 34), a common febrile exanthematous illness. In addition, in transplant recipients, HHV-6B reactivation can cause several clinical manifestations such as encephalitis, bone marrow suppression, and pneumonitis (32). In contrast to HHV-6B, HHV-6A seems to be less prevalent in the population: it is rarely detected in transplant recipients with encephalitis (7, 10), but it has been implicated in the pathogenesis of multiple sclerosis (25). *In vivo* studies have suggested that HHV-6A has a stronger neurotropism than HHV-6B (1, 15). To date, however, neither the clinical features of primary HHV-6A infection nor the full spectrum of diseases associated with HHV-6A have been elucidated.

Methods for the differentiation between HHV-6A and HHV-6B have been developed based on restriction fragment length polymorphism analysis of PCR products, PCR with virus species-specific primers, and Southern blotting with virus species-specific probes (3, 4, 12, 28). Subsequently, real-time PCR methods using virus species-specific primers or probes have been introduced for easier discrimination between the two virus species (6). Recent PCR-based molecular epidemiological analysis demonstrated that HHV-6A is highly endemic in the region of sub-Saharan Africa (5, 18). However, it is difficult to discriminate between active and latent infections on the basis of PCR analysis because these viruses can latently infect peripheral blood mononuclear cells (PBMCs) after primary infection. Furthermore, the most important problem of molecular epidemiological analysis is that this analysis does not reveal precise seroepidemiology and can be affected by the sensitivity of PCR method used. Thus, the lack of a virus species-specific serological assay has hampered the elucidation of clinical features and epidemiology of HHV-6A infection.

The ideal gene target for the development of a virus species-specific serological assay would be a gene with low sequence homology between the two virus species encoding a strong immunoreactive protein. We chose the U11 gene, which encodes a major antigenic structural protein and has 81% amino acid sequence identity between HHV-6A and HHV-6B (11, 17). Previous studies have shown that the 101-kDa HHV-6B virion protein (101K) encoded by the U11 gene is highly immunoreactive in immunoblotting analysis and is a specific serological marker of infection (24, 30). Therefore, we sought to develop a virus species-specific serological assay based on immunoblotting analysis utilizing the U11 gene of HHV-6. The reliability of this novel virus species-specific assay was examined using human sera collected from patients with various types of HHV-6 infection.

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MATERIALS AND METHODS

Cells and viruses. Cord blood mononuclear cells were separated by Ficoll-Hypaque gradient centrifugation from heparinized cord blood samples and stimulated for 2 days before inoculation with the viruses in RPMI 1640 medium containing 20% fetal calf serum, 0.1 U of recombinant human interleukin-2/ml, and 5 μg of phytohemagglutinin-P/ml. HHV-6A (U1102 strain) or HHV-6B (Z29 strain) were propagated in cord blood mononuclear cells. At day 7 postinoculation, when the infected cells showed maximum levels of cytopathic effects, the infected cells were harvested and lysed by repeated freezing and thawing. The samples were stored at −20°C until the generation of expression vectors.

Patients and samples. Four panels of serum samples (total, 87 serum samples) were collected from patients with different clinical symptoms (1). Positive control sera for HHV-6A infection were collected from two patients with chromosomally integrated HHV-6A (ciHHV-6A) and one patient with chronic fatigue syndrome and HHV-6A infection (2). Positive control sera for HHV-6B infection were 12 paired sera obtained from patients with exanthem subitum (age, 7 to 17 months; median age, 11 months). Primary HHV-6B infection was confirmed by virus isolation and seroconversion for IgG HHV-6 antibody (3). As controls for HHV-6B reactivation, six paired sera were obtained from drug-induced hypersensitivity syndrome (DIHS) patients (age range, 29 to 73 years; median age, 61.5 years) and five paired sera from hematopoietic stem cell transplant (HSCT) recipients with HHV-6 reactivation (age range, 10 months to 11 years; median age, 3 years). HHV-6B reactivation was confirmed by virus isolation and serological analysis in all of the patients (4). As control sera for considering as previous infection with HHV-6B, 38 healthy seropositive adult sera (age range, 20 to 88 years; median age, 31 years) were used.

In addition to the serum samples, 24 PBMC and 38 saliva samples were collected from the 38 healthy control adult subjects to assess the presence of latent virus species-specific infection. All samples were stored at −20°C until assayed.

Since patients with primary HHV-6A infection are very difficult to identify, serum obtained from macaques inoculated with HHV-6A (21) was used to determine whether this assay can specifically detect HHV-6A-specific antibodies.

DNA extraction. Viral DNAs were extracted from the HHV-6A (U1102) and HHV-6B (Z29)-infected cord blood mononuclear cells using a QIAamp DNA blood minikit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions and were used as templates for the generation of the expression vectors. In order to determine the genotype of HHV-6, viral DNA was extracted from PBMCs and saliva obtained from 38 seropositive healthy adults using the same DNA extraction kit. Then, extracted DNA was analyzed by nested PCR to discriminate between HHV-6A and HHV-6B.

PCR assay. Details of the nested PCR for determining the genotype of HHV-6 was described in a previous report (28). In brief, the PCR amplifies a 751-bp DNA fragment containing the gene that encodes a putative large tegument protein (U31 gene) using *Taq* DNA polymerase (AmpliTaq Gold; Perkin-Elmer, Norwalk, CT). The identity of the virus species of HHV-6 was determined by the presence or absence of a HindIII site in a second PCR product.

Construction of HHV-6 U11 gene expression plasmid. The U11 genes of HHV-6A and HHV-6B encoding the p100 and 101K proteins, respectively, were amplified with DNA polymerase (2.5 PFU/μl; Fermentas Life Sciences, Maryland) using the following primer sets. The upstream and downstream primers for amplification of the U11 gene of HHV-6A (U1102) were HHV-6A U11 NcoI (5′-GCGATGGCCATGGATATCATGGATCTGCAAAGACAT-3′) and HHV-6 AB U11 SacI (5′-GTCGACGGAGCTCGACGACGCGATCGCTGA-3′). In order to amplify the U11 gene of HHV-6B (Z29), HHV-6B U11 NcoI (5′-GCGATGGCCATGGATATCATGGATTTGAAAGCGCAG) was used for the upstream primer, and HHV-6 AB U11 SacI was used for the downstream primer.

The upstream primer has a NcoI restriction enzyme site, and the downstream primer has a SacI restriction enzyme site (underlining indicates restriction enzyme sites). The PCR products were digested with the restriction endonucleases NcoI and SacI (New England Biolabs, Massachusetts), respectively. The purified fragments were ligated into the histidine-tagged expression vector pET22b(+) (Novagen, California) using Ligation High (Toyobo, Japan). As shown in Fig. 1, the plasmids, pET22b(+)-6AU11 and pET22b(+)-6BU11, consisting of a pelB leader on the N-terminal end and a His_6 tag on the C-terminal end, were used to transform the competent *Escherichia coli* strain Nova Blue (Invitrogen, California).

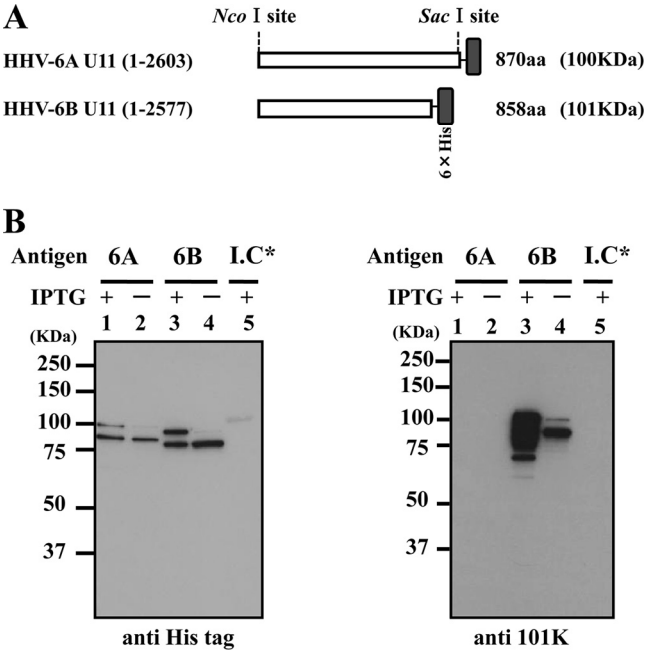

FIG 1 (A) Construction of the HHV-6 U11 gene expression plasmids pET22b(+)-6AU11 and pET22b(+)-6BU11. These recombinant proteins expressed His_6 tag on the C-terminal end. (B) Expression of the recombinant proteins p100 (6A) and 101K (6B) and immunoblot analysis. Induction was carried out using 1 mM IPTG for 3 h at 30°C (+) or without 1 mM IPTG (−). Induction control (I.C*) was expressed by 1 mM IPTG for 3 h at 30°C. Molecular mass standards are indicated in kilodaltons on the left. Detection of recombinant p100 and 101K using an anti-His tag antibody. The anti-His tag antibody reacted with both recombinant p100 (6A) and 101K (6B) (lanes 1 and 3 of the left panel, respectively). Detection of recombinant 101K using an anti-101K antibody. The anti-101K antibody reacted with recombinant 101K (lane 3 of the right panel).

Expression of p100 and 101K. *E. coli* strain BL21 cells (Invitrogen) were transformed using the recombinant pET22b(+)-6AU11 and pET22b(+)-6BU11 plasmids. Recombinant p100 and 101K were expressed by optical induction with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 h at 30°C. The bacteria were harvested, and the cell pellets were frozen at −80°C overnight. The frozen pellets were thawed and resuspended in 200 μl of lysis buffer (phosphate-buffered saline [PBS] with 0.05% NaN_3) supplemented with protease inhibitor (Roche Applied Science, Indiana). The suspensions were sonicated, and the extracts were used for immunoblot analysis.

Immunoblot assay. Recombinant p100 and 101K were resolved by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE), and electrophoretically transferred onto a polyvinylidene difluoride membrane (ATTO, Tokyo, Japan). The membrane was incubated in blocking buffer (5% skim milk in PBS) overnight at 4°C. Next, the membrane was incubated for 1 h at room temperature with the primary antibodies mouse anti-His tag antibody (MBL, 6C4, M089-3) and mouse anti-101K (Chemicon, MAB8535, C3108-103) and diluted to 1:1,500 using antibody dilution buffer (5% skim milk in PBS). Meanwhile, human serum samples were diluted to 1:500 for use as primary antibodies. A

preliminary experiment for determining an appropriate dilution of serum samples was carried out, and finally a 500 times dilution of serum sample was the optimal dilution for this immunoblot (IB) assay. After the membrane was washed with PBS, it was incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (MBL, Nagoya, Japan) or HRP-conjugated mouse anti-human IgG (MBL) antibodies. The enzyme-labeled antibodies were detected by using an ECL Plus Western blotting detection system (GE Healthcare Biosciences, United Kingdom) according to the manufacturer's instructions.

IFA. Antibody titers to HHV-6 were measured by indirect immunofluorescence assay (IFA) as described previously. The detection limit of the IFA titer was <8 (34). Representative strains of HHV-6A (U1102) and HHV-6B (Z29) were used as the standard antigens. The antibody titer was defined as the reciprocal of the serum dilution showing specific fluorescence.

RESULTS

Expression of HHV-6A and HHV-6B U11 gene-encoded proteins. To determine whether the HHV-6A and HHV-6B U11 gene expression plasmids [pET22b(+)-6AU11 and pET22b(+)-6BU11] expressed the expected proteins, immunoblotting analysis was carried out with an anti-His tag antibody and an anti-101K antibody (Fig. 1B). As expected, the pET22b(+)-6AU11 product was recognized as a 100-kDa band, and the pET22b(+)-6BU11 product was recognized as a 101-kDa band using the anti-His tag antibody. Moreover, only the pET22b(+)-6BU11 product was detected by the anti-101K HHV-6B-specific monoclonal antibody, demonstrating HHV-6B-specific protein expression. Since an HHV-6A U11 gene product-specific monoclonal antibody was not available, it was impossible to determine whether the pET22b(+)-6AU11 product was virus species specific. As shown in Fig. 1B, these plasmids synthesized the specific proteins without IPTG induction, and not only was the upregulation of protein synthesis demonstrated but also the production of additional proteins after IPTG induction.

Evaluation of the assay for clinical applications. Assessment of HHV-6A-infected patients (no information with regard to HHV-6B infection) revealed that two of the three sera (patients 2 and 3) reacted with both p100 and 101K (Table 1). Representative results of immunoblotting analysis in patients 2 and 3 are shown in Fig. 2A and B, respectively. Serum collected from patient 1, a ciHHV-6A patient with central nervous system manifestations, did not react with either protein despite having low titers of HHV-6A and HHV-6B antibodies based on IFA analysis.

None of the acute-phase sera from 12 patients with primary HHV-6B infection (exanthem subitum) reacted with either p100 or 101K (Table 1 and Fig. 2C). However, 10 of the 12 (83.3%) convalescent-phase sera reacted exclusively with 101K (Table 1 and Fig. 2C). In contrast to the immunoblotting results, seroconversion against both HHV-6A and HHV-6B was demonstrated in all 12 patients by IFA analysis, reflecting the presence of cross-reactive epitopes. As shown in Table 1, the two negative sera were collected on days 10 (patient 8) and 11 (patient 7) after the onset of the illness and showed relatively low IFA antibody titers. Unexpectedly, monkey sera obtained from macaques inoculated with HHV-6A were found to be reactive with both p100 and 101K (data not shown). It is not clear whether this finding reflects the presence of preexistent antibodies against a monkey variant of HHV-6B or a cross-reaction between A and B antibodies.

Sera from DIHS patients with HHV-6 reactivation revealed reactivity with 101K in only three of the six acute-phase sera (50.0%). However, all convalescent-phase sera reacted with 101K (Table 1), and two of them (patients 16 and 17) reacted not only with 101K but also with p100 (Table 1). In contrast, all acute- and convalescent-phase sera collected from HSCT recipients reacted exclusively with 101K (Table 1).

In healthy adults considered to have previous infection with HHV-6B, 31 of the 38 (81.6%) sera were positive for 101K-specific antibodies (Table 2 and Fig. 2D). Three (patients 21, 26, and 33) of the seven negative sera contained low titers of HHV-6B IgG antibodies (×8) as determined by IFA, while in the remaining four sera the IFA titers of HHV-6B IgG antibodies were 1:16. Four (patients 5, 25, 28, and 29) of the 38 sera (10.5%) reacted with both 101K and p100 (Table 2 and Fig. 2E). In order to determine whether these subjects were latently infected with the two HHV-6 virus species, detection of viral DNA from PBMCs or saliva was carried out using nested PCR and genotyping (Table 2). HHV-6B DNA was detected in 9 (37.5%) of the 24 PBMC samples and in 27 (71.1%) of the 38 saliva samples. None of the samples contained HHV-6A DNA. Four (patients 8, 9, 26, and 33) of the seven cases without p100 and 101K antibodies contained HHV-6B DNA in either PBMCs or saliva. In addition, only HHV-6B DNA was detected in either PBMCs or saliva collected from three (patients 5, 28, and 29) of the four cases having both p100 and 101K antibodies, while the fourth was negative for both HHV-6A and HHV-6B DNA.

DISCUSSION

The development of a virus species-specific serologic assay for HHV-6A and HHV-6B antibodies is urgently needed in order to evaluate the full pathogenic potential of the two virus species. Using the U11 gene products p100 (HHV-6A) and 101K (HHV-6B), we developed a novel assay that permits the differentiation of antibodies directed against these two viruses. Quality controls demonstrated that recombinant p100 (HHV-6A) and 101K (HHV-6B) were correctly expressed by immunoblot analysis and that 101K was HHV-6B specific. Although it is important to note that it was impossible to prove that the p100 antigen was HHV-6A specific because no appropriate monoclonal antibody is currently available, specificity was demonstrated using human sera from patients with primary HHV-6B infection (exanthem subitum) which failed to cross-recognize the p100 antigen. In contrast, we were unable to discriminate between primary HHV-6A and HHV-6B infections by IFA assay, even though the IFA assay was more sensitive than the immunoblotting assay. This is likely due to the fact that IFA utilizes whole virus as an antigen, which contains multiple cross-reactive epitopes between the two virus species. These findings suggest that our immunoblotting assay could be useful for distinguishing between primary HHV-6A and HHV-6B infections; however, the assay sensitivity could be improved in future developments.

One of the two sera collected from patients with ciHHV-6A and one serum collected from a chronic fatigue patient with persistent HHV-6A infection were found to react with both p100 and 101K antigens. However, neither p100 antibodies nor 101K antibodies were detected in the serum obtained from one of the ciHHV-6A patients. Since IFA antibody titers against HHV-6A and HHV-6B were relatively low in this patient, in comparison to the other two patients, it is possible that the patient (patient 1) had low levels of HHV-6A or HHV-6B antibodies that were below the detection limit of immunoblotting analysis. Although it has been suggested that ciHHV-6 patients develop HHV-6 antibodies (14, 19, 23), to our knowledge this is the first report examining virus

TABLE 1 Demographics, clinical features, serological results based on immunoblotting using recombinant p100 or 101K, and HHV-6 IFA titer for patients in this study

Patient	Diagnosis[a]	Sex[b]	Age	Sampling days	IB[c]		IFA titer[d]	
					p100	101K	6A	6B
1	CI-HHV-6A	M	60 yr		–	–	16	8
2	CI-HHV-6A	M	13 yr		+	+	64	64
3	CFS	M	56 yr		+	+	128	128
4	ES	M	13 mo	0	–	–	<8	<8
				9	–	+	64	>256
5	ES	M	9 mo	6	–	–	<8	<8
				14	–	+	128	128
6	ES	F	15 mo	4	–	–	<8	<8
				32	–	+	128	128
7	ES	M	13 mo	3	–	–	<8	<8
				11	–	–	32	32
8	ES	M	17 mo	1	–	–	<8	<8
				10	–	–	8	64
9	ES	M	12 mo	4	–	–	<8	<8
				21	–	+	32	>256
10	ES	F	10 mo	0	–	–	<8	<8
				12	–	+	>256	32
11	ES	F	11 mo	2	–	–	<8	<8
				18	–	+	64	64
12	ES	M	9 mo	3	–	–	<8	<8
				17	–	+	128	128
13	ES	F	8 mo	2	–	–	<8	<8
				12	–	+	64	64
14	ES	F	7 mo	2	–	–	<8	<8
				12	–	+	32	64
15	ES	M	8 mo	3	–	–	<8	<8
				15	–	+	128	>256
16	DIHS	M	29 yr	12	–	+	<8	8
				37	+	+	256	>256
17	DIHS	M	34 yr	9	–	+	<8	8
				29	+	+	16	64
18	DIHS	M	66 yr	7	–	–	<8	8
				43	–	+	16	128
19	DIHS	M	64 yr	19	–	–	<8	16
				45	–	+	128	>256
20	DIHS	F	59 yr	21	–	+	<8	8
				38	–	+	32	128
21	DIHS	M	73 yr	9	–	–	<8	<8
				27	–	+	8	128
22	HSCT	M	3 yr	8	–	+	32	16
				43	–	+	64	256
23	HSCT	M	10 mo	13	–	+	<8	8
				62	–	+	256	256
24	HSCT	F	1 yr	3	–	+	16	128
				31	–	+	64	128
25	HSCT	M	11 yr	6	–	+	<8	16
				26	–	+	32	128
26	HSCT	M	3 yr	15	–	+	32	32
				50	–	+	256	256

[a] CIHHV-6A, chromosomally integrated HHV-6A; CFS, chronic fatigue syndrome; ES, exanthem subitum; DIHS, drug-induced hypersensitivity syndrome; HSCT, hematopoietic stem cell transplant.
[b] M, male; F, female.
[c] IB, immunoblot assay using p100 or 101K; −, No reactivity; +, reactivity.
[d] The detection limit of the IFA was <8.

species-specific antibodies in ciHHV-6 patients. Not only p100-specific but also 101K-specific antibodies were detected in patient 2, in whom the HHV-6A genome was integrated. One possible explanation for the detection of 101K-specific antibodies is that the patient was previously infected with HHV-6B. To confirm the hypothesis, ciHHV-6 patients should be prospectively monitored to determine whether they could be infected with another HHV-6 virus species.

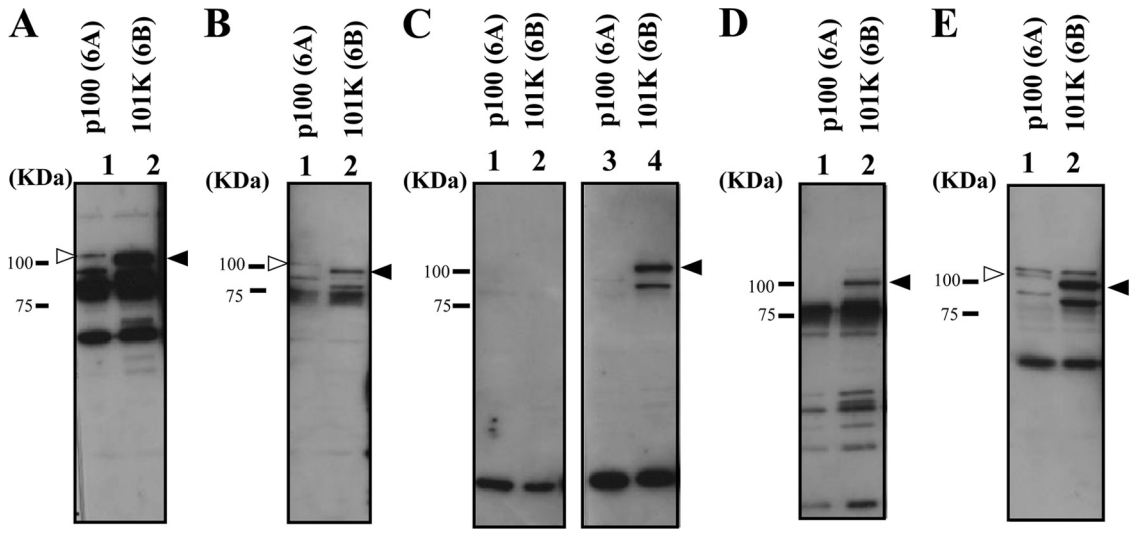

FIG 2 Immunoblot analysis with human sera collected from patients with various types of HHV-6 infections against recombinant p100 and 101K antigens. All human sera were diluted to 1:500 for the assay. The patient sera collected from a ci-HHV-6A patient (patient 2 [A]) and a CFS patient (patient 3 [B]) reacted with p100 (lane 1) and 101K (lane 2). (C) Paired sera obtained from exanthem subitum (primary HHV-6B infection) were used for the assay. Acute-phase serum did not react with p100 (lane 1) and 101K (lane 2). Meanwhile, convalescent-phase serum reacted with only 101K (lane 4). A healthy adult serum (patient 6) reacted with only 101K (D), and another healthy adult serum (patient 26) reacted with both p100 and 101k (E). Molecular mass standards are indicated in kilodaltons on the left. An open arrowhead indicates a specific band reacted to the p100 antigen. A solid arrowhead indicates a specific band reacted to the 101K antigen.

At present, if a patient has both p100 and 101K antibodies, on the basis of our immunoblotting assay, there are two possible explanations: (i) a cross-reaction between HHV-6A and HHV-6B antibodies (a patient infected with only HHV-6B) has occurred or (ii) a double infection of HHV-6A and HHV-6B (a patient infected with two viruses) has occurred. In order to perform seroepidemiological analysis, it is critical to determine whether p100 antigen is HHV-6A specific or not. Thus, serum obtained from patients with primary pure HHV-6A infection would be necessary to evaluate the specificity of this assay for detection of HHV-6A antibodies; indeed, such patients are difficult to identify, except possibly among children in regions where HHV-6A is endemic (5). A recently reported microwell adapted immunoblot assay for HHV-6 species-specific serological analysis contains same problem. In that study the researchers used pooled serum samples collected from adult and child donors to test assay reliability. Since the infectious status of the HHV-6 species in the subjects was not clear, it is impossible to be certain whether the assay was virus species specific or not (29).

Instead of serum collected from patients with primary HHV-6A infection, serum samples collected from macaques inoculated with HHV-6A were used to determine whether p100 antigen contains an HHV-6A-specific epitope. In contrast to our expectations, the monkey serum reacted with both p100 and 101K. In addition to the possibility of cross-reactivity between HHV-6A and HHV-6B, since the presence of antibodies to HHV-6 has previously been demonstrated in macaques (16), it is possible that an HHV-6B-like virus persistently infects macaques, inducing cross-reactive antibodies that were detected by the present assay. Although serum collected from macaques before virus inoculation would be useful for this experiment, no such samples was available because pooled serum samples obtained in the previous study were used in this experiment.

All 10 paired sera obtained from HSCT recipients with HHV-6B reactivation reacted exclusively with 101K antigen. According to the seroepidemiological studies conducted in Japan (33, 35), most transplant recipients are HHV-6 seropositive. A previous molecular epidemiological study demonstrated that all HHV-6 isolates recovered from transplant recipients in Japan were HHV-6B (13). Thus, the present findings are consistent with previous seroepidemiological and molecular epidemiological studies. In contrast to transplant recipients, three of the six acute-phase serum samples obtained from DIHS patients did not contain 101K-specific antibodies. As suggested in previous studies (22, 26), sera from acute-phase DIHS patients appeared to have relatively low levels of HHV-6 IgG antibodies, as measured by the IFA assay. Thus, the three DIHS patients might have had low levels of 101K-specific antibodies, which were below the detection limit of the present immunoblotting assay. Interestingly, although the isolates from peripheral blood of the two DIHS patients were HHV-6B, seroconversion against p100 antigen was observed in these patients. This finding suggests both that HHV-6A reactivation might occur in anatomic sites (e.g., the salivary glands or central nervous system) other than the peripheral blood, which have been suggested as sites of HHV-6 latency (1, 6, 14, 15), and that HHV-6B might be cross-reactive.

Of the 38 (81.6%) serum samples collected from healthy adults, 31 reacted with 101K. The seven negative serum samples had low levels of IgG antibodies ($\times 8$ to $\times 16$), as measured by IFA. Thus, it is likely that the seven negative sera contained low levels of HHV-6B-specific antibodies that were below the detection limit of our assay. Since HHV-6B DNA was detected in four (patients 8, 9, 26, and 33) of the seven cases without both antibodies, nested PCR, which detects latently infected HHV-6B, is likely to be more sensitive than our immunoblotting assay. Of the 38 serum samples, 4 (10.5%) also reacted with the p100 antigen. Molecular epidemiological studies have suggested that the prevalence of ciHHV-6A population is low both

TABLE 2 Demographics, clinical features, serological results based on immunoblot using recombinant p100 or 101K, HHV-6 IFA titer, and PCR genotyping among healthy adults

Healthy adult	Sex[a]	Age (yr)	IB[b]		IFA titer[c]		Nested PCR[d]	
			p100	101K	6A	6B	PBMCs	Saliva
1	M	47	–	–	<8	16	–	–
2	F	44	–	+	32	32	–	B
3	M	23	–	+	16	32	–	B
4	M	46	–	+	16	16	B	B
5	F	20	+	+	32	32	–	B
6	F	23	–	+	<8	8	–	B
7	F	24	–	+	32	32	B	B
8	F	22	–	–	8	16	B	B
9	M	22	–	–	16	16	–	B
10	M	22	–	+	16	32	–	B
11	F	22	–	+	8	16	–	–
12	M	24	–	+	64	64	–	B
13	F	23	–	+	16	32	B	B
14	M	23	–	+	8	16	–	B
15	F	24	–	+	32	32	–	B
16	M	23	–	+	16	16	–	B
17	F	23	–	+	64	64	B	B
18	M	24	–	+	16	16	B	B
19	M	25	–	+	<8	8	B	B
20	F	30	–	+	16	64	B	B
21	M	38	–	–	<8	8	–	–
22	F	23	–	+	8	16	–	B
23	M	24	–	+	8	32	B	B
24	M	32	–	+	8	32	–	–
25	F	78	+	+	8	16	ND	–
26	F	75	–	–	<8	8	ND	B
27	F	73	–	+	8	16	ND	B
28	M	86	+	+	<8	8	ND	B
29	M	69	+	+	16	32	ND	B
30	F	62	–	+	<8	8	ND	–
31	F	73	–	+	<8	8	ND	–
32	F	88	–	+	32	64	ND	–
33	F	78	–	–	<8	8	ND	B
34	F	82	–	–	<8	16	ND	–
35	F	72	–	+	8	32	ND	B
36	M	60	–	+	<8	<8	ND	–
37	M	68	–	+	<8	<8	ND	B
38	M	72	–	+	<8	16	ND	–

[a] M, male; F, female.
[b] Samples were tested by immunoblot assay (IB) using p100 or 101K. −, No reactivity; +, reactivity.
[c] The detection limit of the IFA was <8.
[d] −, Negative result; B, HHV-6 variant B; ND, not determined because there was no sample.

in Western countries (14) and in Japan (27). However, recent reports have demonstrated a high prevalence of HHV-6A in sub-Saharan Africa (5, 18). Although a molecular epidemiological examination was carried out in PBMCs and saliva obtained from our healthy subjects, no HHV-6A was identified in any of these samples. Most of the previous molecular epidemiological studies examining PBMCs or saliva detected a high frequency of latent HHV-6B infection (1, 8). However, it has been suggested that mixed infection of the two virus species frequently occurs in lung tissues (9); thus, we speculate that persistent infection with HHV-6A might occur in the lung tissues of patients with p100 antibody.

In conclusion, although further serological analysis using samples collected from patients with primary HHV-6A infection is necessary, we have developed a new immunoblot assay that reliably identifies antibodies to HHV-6A and HHV-6B. Further seroepidemiological studies using the immunoblot analysis in combination with molecular epidemiological study will be necessary to clarify the full spectrum of HHV-6A infection. In addition, it will be important to develop an enzyme-linked immunosorbent assay using these recombinant proteins for a high-throughput analysis of samples.

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